UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

Influence of metabolic stress in bovine fetal development: an allometric study

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To my loving siblings who always encouraged me

To my parents for the all the love, constant support and never ending patience.

Resumo

Efeitos do stress metabólico no desenvolvimento do feto bovino: estudo sobre alometria

Durante o período de transição as vacas de leite enfrentam enormes alterações metabólicas com repercussões na sua saúde e produtividade. Face à possibilidade de que o stress nesta última fase da gestação pode também influenciar o feto, ou até mesmo ter efeitos secundários que irão afetar o vitelo, esta é uma área de estudo que não pode ser ignorada.

O objetivo deste estudo pretende determinar se, na presença de stress metabólico materno, há prioridade no desenvolvimento de órgãos vitais no feto, como o cérebro e o coração em específico, sobre o desenvolvimento dos ossos longos, mais precisamente o rádio-ulna e o metatarso. Adicionalmente as diferenças entre países, particularmente ambientais, foram consideradas como um possível fator agravante do stress metabólico que, consequentemente, poderia realçar diferenças na alometria dos órgãos em estudo.

Para os propósitos deste estudo, várias medições foram realizadas em 171 vitelos recém-nascidos de raça Holstein Frísia com um medidor ósseo e uma fita métrica. Foram medidos o diâmetro e a circunferência da cabeça (HD e HC, respetivamente), a circunferência do peito (HG), o comprimento do antebraço e do metatarso (FL e ML, respetivamente), a largura das ancas e dos ombros (HW e SW, respetivamente), a altura (WH) e o comprimento diagonal (DL). Os vitelos em questão foram medidos nas respetivas explorações, uma exploração de vacas leiteiras em Portugal e duas na Bélgica. Também foram recolhidos outros dados provenientes dos registos das explorações como a produção leiteira do ano (M305d), a paridade e a gemelaridade das mães, e a época e temperatura em que foram realizadas as medições.

Os rácios das medições (HC/ML, HC/FL, HD/ML, HD/FL, HG/ML e HG/FL) foram utilizados como indicadores de alometria dos órgãos fetais em estudo e analisados em relação aos dados obtidos das vacas. Algumas conexões significativas ($P<0.05$) foram evidenciadas nos vitelos belgas, entre os rácios com a paridade e a época de medição. Os rácios demonstram uma tendência em crescer com o aumento dessas duas variáveis. No entanto, os vitelos portugueses não comprovaram nenhum destes resultados. Esta discrepância entre as análises dos vitelos portugueses e belgas, provavelmente causada por uma amostra insuficiente, levam-nos a crer que as conclusões tiradas deste estudo são prematuras e que deveriam ser realizados estudos adicionais de modo a esclarecê-las.

Subsequentemente, a relação entre o stress metabólico e o desenvolvimento do feto também se mantém incerta e deverá continuar a ser investigada.

Palavras-chave: stress metabólico, período de transição, balanço energético negativo, vacas leiteiras, alometria

Abstract

Influence of metabolic stress in bovine fetal development: allometry study

Dairy cows go through great metabolic change during the transition period, with several detrimental side effects on health and productivity. The possibility that the stress they undertake on the final phase of gestation can also influence the unborn calf or even have carryover effects that will impact him after birth is not one that should be ignored.

The main goal of this study was to determine if calves born from metabolic stressed cows prioritized the development of vital organs, brain and heart specifically, over the development of the long bones, more precisely the radius-ulna and the metatarsus. Additionally, differences between countries, particularly environmental, were also considered as a possible aggravator of metabolic stress and, consequently, of allometric fetal development.

For the purposes of this study, the head diameter and circumference (HD and HC, respectively), chest circumference (HG), forearm and metatarsal lengths (FL and ML, respectively), hip and shoulder width (HW and SW, respectively), height (WH) and diagonal length (DL) of 171 newborn Holstein Friesian calves from one farm in Portugal and two farms in Belgium were measured with callipers and a measuring tape. The dam's milk production for the year (M305d), parity and gemelarity, as well as the season when the measurements were performed, were also registered.

Measurement ratios (HC/ML, HC/FL, HD/ML, HD/FL, HG/ML and HG/FL) were used as an indicator of prenatal allometric growth and analysed against the data obtained from the mothers. Some significant correlations were evidenced ($P<0.05$) in the belgian calves, between the ratios with parity and season, demonstrating a tendency towards higher ratios with the increase of these two variables. However, the portuguese calves supported none of these results. This discrepancy obtained from the analysis of the portuguese and belgian calves, probably the result of an insufficient sample size, led us to believe that the conclusions drawn from this study are most likely premature and that further studies should be conducted in order to clarify them.

Subsequently, the relation between metabolic stress and fetal development also remains unclear and should be the subject of further investigation.

keyword: metabolic stress, transition period, negative energy balance, dairy cattle, allometry

Resumo II

Efeitos do stress metabólico no desenvolvimento do feto bovino: estudo sobre alometria

Ao longo de várias décadas as vacas leiteiras têm vindo a ser selecionadas com o objetivo de aumentar a sua produtividade. Esta seleção apesar de ter trazido grandes benefícios a nível económico para a indústria do leite, apresenta também diversas consequências para a saúde e bem-estar destes animais. A prioridade que é dada ao transporte de nutrientes para a glândula mamária, de modo a suportar o aumento do volume produtivo, ocorre em detrimento do aporte de nutrientes para os restantes tecidos. Este aumento da energia despendida em produção, para além de significar uma redução na energia disponível para satisfazer as necessidades de manutenção do animal, ocorre em simultâneo com o decréscimo na ingestão de matéria seca que ocorre no periparto. Esta discrepância entre as necessidades energéticas do animal e a energia disponível para as preencher, é resolvida com recurso a uma série de alterações metabólicas e uma utilização particular dos nutrientes provenientes da dieta e das reservas energéticas presentes no tecido adiposo. Estes processos de adaptação requerem um balanço minucioso entre os diversos substratos energéticos (glucose, ácidos gordos não esterificados e corpos cetónicos), sendo que quaisquer desequilíbrios no seu uso e mobilização podem resultar em stress metabólico para o animal.

A ligação que o stress metabólico tem com a grande incidência de doenças durante a época do periparto já se encontra bem estabelecida, no entanto, pouco se sabe sobre o possível efeito que ele pode ter na gestação. Face à possibilidade de que o stress nesta última fase da gravidez pode também influenciar o feto, ou até mesmo ter efeitos secundários que irão afetar a saúde e a produtividade do vitelo, esta é uma área de estudo que não pode ser ignorada.

Com o propósito de expandir o nosso conhecimento sobre este tópico, foi realizado este estudo com o objetivo de determinar se na presença de stress metabólico materno, há prioridade no desenvolvimento de órgãos vitais no feto, como o cérebro e o coração em específico, sobre o desenvolvimento dos ossos longos, mais precisamente o rádio-ulna e o metatarso. Adicionalmente, foram utilizadas amostras de países distintos numa tentativa de determinar se quaisquer discrepâncias entre os resultados desses países, poderiam ser atribuídas à influência de fatores ambientais. Mais especificamente, se a possível presença de stress térmico, derivado da diferença de temperatura nestes países durante a realização do estudo, poderia influenciar o grau de stress metabólico dos animais e refletir-se em diferenças na alometria dos órgãos em estudo.

Este estudo teve como amostra 171 vitelos recém-nascidos de raça Holstein Frísia provenientes de 3 explorações, sendo 100 vitelos de uma exploração em Portugal e 71 vitelos

de duas explorações belgas. Utilizando um medidor ósseo e uma fita métrica, várias medições foram feitas nos vitelos de modo a determinar o crescimento dos órgãos vitais e dos ossos longos. Todo o protocolo foi padronizado o máximo possível, com o objetivo de potenciar a facilidade da sua replicação nos vitelos recém-nascidos. A dimensão dos órgãos vitais foi determinada indiretamente através da medição das estruturas ósseas que os protegem. Assim para o cérebro foi determinada a dimensão do crânio, com o diâmetro e a circunferência da cabeça (HD e HC, respetivamente), e para o coração foi determinada a dimensão do tórax, com a circunferência do peito (HG). Em contrapartida, a dimensão dos ossos longos foi determinada diretamente com a medição do comprimento do antebraço e do metatarso (FL e ML, respetivamente). Foram ainda medidas a largura das ancas (HW), a largura dos ombros (SW), a altura a nível da cernelha (WH) e o comprimento diagonal (DL).

O stress metabólico materno também foi determinado indiretamente, com recurso ao registo produtivo das explorações. Assim foram recolhidos os dados relativos à produção leiteira do ano (M305d) e à paridade e gemelaridade das mães. Finalmente a época e a temperatura quando foram realizadas as medições também foram registadas. Para os vitelos belgas época 1 (fevereiro 2017) e época 2 (março 2017), com uma temperatura média de 4.5 ºC e 5.3 ºC, respetivamente, e para os vitelos portugueses época 3 (julho e agosto de 2017) com uma temperatura média de 25 ºC.

Utilizando as medições dos órgãos vitais e dos ossos longos foram calculados rácios representativos da relação alométrica destes órgãos (HC/ML, HC/FL, HD/ML, HD/FL, HG/ML e HG/FL). Posteriormente estes rácios foram analisados com os indicadores de stress metabólico materno com testes de regressão linear de modo a determinar a presença de alguma relação significativa entre eles.

Restringindo-nos à amostra proveniente da exploração portuguesa, muito pouco foi possível deduzir dos resultados obtidos. Apenas se determinou a presença de dimensões significativamente superiores ($p<0.05$) do diâmetro da cabeça (HD), da altura (WH) e da largura dos ombros (SW) em vitelos machos, e novamente, do diâmetro da cabeça (HD) para paridades superiores a seis. Nenhum resultado significativo surgiu entre os rácios com as restantes variáveis.

Em contrapartida a amostra proveniente das explorações belgas surtiu uma maior quantidade de resultados significativos. Diversos rácios (HD/ML, HD/FL, HG/ML e HG/FL) apresentaram resultados significativamente superiores ($p<0.05$) na transição de paridade 1 para paridade 2. Adicionalmente, todos os rácios apresentaram um aumento significativo ($p<0.05$) da primeira para a segunda época de medição e, consequentemente, da temperatura correspondente à época 1 (4.5 ºC) para a temperatura correspondente à época 2 (5.3 ºC).

As presenças destes resultados nos vitelos belgas permitiram retirar algumas conclusões, todavia, a discrepância entre os resultados obtidos das duas amostras,

portuguesa e belga, sugere que as deduções tiradas neste ensaio são prematuras e que estudos adicionais devem ser realizados de modo a esclarecê-las.

Finalmente, com o intuito de analisar ambas as amostras como um todo, foi realizada uma análise de variância entre elas, que revelou a presença de diferenças significativas entre a amostra portuguesa e a amostra belga. Análises subsequentes apenas reforçaram esta diferença, levando à conclusão de que as amostras eram demasiado distintas para serem analisadas em conjunto.

Numa tentativa de justificar esta discrepância entre amostras foi testada a sua relação com as restantes variáveis em estudo. Isso permitiu determinar que, entre essas variáveis, apenas a época de medição apresentava algum peso significativo, capaz de justificar a diferença de variância entre a amostra portuguesa e a amostra belga. No entanto também foram consideradas outras possibilidades como as diferenças entre explorações não quantificadas neste estudo (a nutrição, as instalações e o maneio), diferenças na técnica de medição (que apesar de utilizar o mesmo protocolo foi realizada por operadores diferentes nos dois países) e diferenças genéticas (porque apesar de todos os animais em estudo pertencerem à raça Holstein Frísia, existe a possibilidade dos dois países estarem a selecionar com objetivos distintos, resultando em animais com atributos diferentes).

No decorrer deste estudo, várias limitações foram detetadas que contribuíram para a escassez de resultados conclusivos. A realização deste protocolo de medições em associação com uma quantificação mais controlada das restantes variáveis, utilizando uma amostra maior e durante um período de tempo superior pode ser necessária de modo a retirar conclusões aplicáveis a toda a raça.

Não obstante, é possível concluir que a relação entre o stress metabólico, a gestação e o desenvolvimento do feto apresenta ainda muitas questões por responder. De modo a potenciar a saúde e bem-estar destes animais, bem como a sua capacidade produtiva, o funcionamento e os mecanismos envolvidos nesta relação devem continuar a ser investigados.

Palavras-chave: stress metabólico, período de transição, balanço energético negativo, vacas leiteiras, alometria

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BCS – body condition score
BHB - beta hydroxybutyrate
CPT I - carnitine palmityl transferase I
DL – diagonal length
DMI - dry matter intake
HC - head circumference
HD - head diameter
HG - heart girth
HW – hip width
IGF(s) - insulin like growth factor(s)
IGF-1 - insulin like growth factor 1
IGF-2 - insulin like growth factor 2
IUGR – intrauterine growth retardation
ML - metatarsus length
NEB - negative energy balance
NEFA(s) - non esterified fatty acid(s)
OAA - oxaloacetate or oxaloacetic acid
PUFA(s) – polyunsaturated fatty acid(s)
FL - forearm length
ROS - reactive oxygen species
SW – shoulder width
TNF-α - tumor necrosis factor α
VFA - volatile fatty acids
WH – withers height

1. Internship Log

During the course of my internship, I strived to obtain the most well-rounded experience possible. To that end I learned and was supervised by veterinary doctors with different approaches or that specialize in different areas of ruminant clinics.

Part of my internship was completed in Portugal, from 5/09/2016 to 29/11/2016, and then continued in Belgium, where I was part of a rotation program in ruminant clinics from 15/02/2017 to 15/05/2017.

I officially began my internship on the 5th of September 2016 with Dr. Pedro Lima who performed ambulatory clinics with a main focus on cattle reproduction. During this time, we performed several pregnancy diagnoses by rectal palpation, artificial inseminations and fertility evaluation protocols.

From the 3rd of October to the 29th of November, I interned with Dr. Dário Guerreiro who also had an ambulatory practice. During this time, I had the opportunity to experience a multitude of activities from urgent cases, such as dystocias, to more standard herd vaccinations. Furthermore, we followed some cases which required field surgical intervention such as a right abomasum displacement and an exploratory laparotomy (that resulted in the detection of a cecal torsion).

Additionally, in the final three weeks of January 2017 (9th to 27th) I was also allowed to work with Dr. José Alface in the dairy farm Fonte de Leite. This allowed me to get a better grasp of the day-to-day vet work performed on a dairy farm. During those weeks, I assisted in reproductive protocols (from echographic pregnancy diagnosis to estrus synchronizations), collecting and screening milk samples for mastitis detection and quality control, vaccination protocols in both cows and calves, and the resolution of any clinical cases that turned up.

In February I started a 3 months' Erasmus internship in the Faculty of Veterinary Medicine in Ghent, Belgium. Each week I rotated among several different specialties in the Ruminant clinics: Internal Medicine, Surgery, Pathology, Reproduction and Ambulatory Medicine. I observed and assisted in many cases during this time, the reproduction rotation specifically was very rewarding due to the high number of cesarians they performed of which I'd only seen a couple before. In the surgical rotation among others, I observed the correction of several birth defects that are common in Belgian Blue calves (such as flexural deformities and prophylactic tracheostomies to prevent recurring throat necrobacillosis). The period with the Pathology team was a good refresher course in clinical necropsies of farm animals. Both internal medicine and ambulatory rotations had a wide array of cases that ranged from simple hoof trimmings to more complex cases.

A more detailed description of the activities I did during my time with Dr. Dário Guerreiro and the clinical rotation in Ghent can be seen, respectively, in tables 1 and 2.

Table 1. Activities performed in ambulatory clinic with Dr. Dário Guerreiro.

Activity	Number of cases
Vaccinations	
Enterotoxaemia's	13 herds
IBR	5 herds
BVD	2 herds
Blue tongue	1 herd
Reproduction	
Pregnancy diagnostics	14 herds
Dystocias	7
Cesarians	2
Andrological exams	6
Clinical cases in calves	30 sick calves
Neonatal diarrheas	18
Pneumonia	9
Anorexia	2
Bloating	1
Clinical cases in cows	37 sick cows
Pneumonias	9
Digestive symptoms	13
Post-partum infections	10
Placental retention	1
Fasciolosis	4
Podal disease	2
Double sole	1 (cow)
Tyloma	1 (bull)
Clinical cases in small ruminants	19 sick small ruminants
Digestive symptoms	10
Keratoconjunctivitis	7
Mastitis	1
Agenesis of teat canal	1
Surgeries	3 field surgeries
Right displaced abomasum	1
Exploratory laparotomy	1 (cecal torsion)
Castration	1
Cesarian	2

Table 1 (continuation). Activities performed in ambulatory clinic with Dr. Dário Guerreiro.

Necropsies	2
Bovine	1
Ovine	1
Euthanasia	1 (equine)

Table 2. Activities performed during the clinical rotation program at Ghent University.

Rotation	Number of cases
Reproduction	**3 weeks**
Physical exam of pregnant cows	Regularly performed 3 times a day
Cesarians	3
Internal Medicine	**2 weeks**
Physical exam and care of newborn ruminants	Regularly performed 3 times a day
Physical exam of sick ruminants	Regularly performed 3 times a day
Surgery	**2 weeks**
Tracheostomy	3
Digit amputation	1
Atresia ani	1
Correction of flexor tendon deformity	1
Ambulatory Clinic	**2 weeks**
Cesarians	5
Podal disease	2 (1 white line defect, 1 double sole)
Euthanasia	1
Castration	2 (rams)
Dehorning	1 (herd)
Sick heifer	1
Pneumonia	1
Pathology	**1 week**
Ruminant necropsies	5

2. Introduction

Over the last half century dairy cattle has been genetically selected in order to produce increasingly larger quantities of milk. However, this rise in milk output did not come without downsides (Gordon 2004). Around calving, a "*transition from the pregnant non-lactating state to the non-pregnant lactating state*" (Goff and Horst 1997, p.1260) presents severe metabolic challenges for the high producing cow. A marked negative energy balance (NEB), due to the disparities between the energetic needs and supply during this transition is common in high yield cows. In the face of this, several metabolic adaptations are employed to help the cow cope until a time when its energetic needs can be fully met (Drackley 1999).

The allocation of glucose is the main nutritional challenge dairy cattle has to contend with. Being indispensable for both fetal and mammary tissues, its prioritization towards them means the rest of the body has to struggle to match its metabolic needs. The mobilization of the body's own natural reserves, fat and muscle, is therefore essential to manage all the demands, while simultaneously allowing glucose to be spared for gestation and lactation (Bell 1995). However, extensive use of these alternative fuels has its own set of repercussions. Aside from being directly linked to metabolic diseases such as fatty liver and ketosis (Herdt 2000), the strain these partitioning mechanisms place in the metabolism to keep up with its fastidious demands, are most likely responsible for the host of peripartum infections and conditions that dairy cattle are subject to (Goff and Horst 1997).

In the past decade, more and more studies (Kessel et al. 2008; LeBlanc 2012; Sordillo and Raphael 2013; Esposito et al. 2013; Sheldon et al. 2018) have tried to unravel the intricacies of metabolic stress and their relation to the vulnerability dairy cattle displays near calving yet, very little has been investigated regarding the potential influence this condition can have on the unborn fetus (Ling et al. 2018). Studies performed on different *in utero* stressors have shown that the consequences of their effects can sometimes still be felt after birth and may affect health, fertility and productivity outcomes of future replacement heifers (Monteiro et al. 2014, 2016; Dahl et al. 2016; Guo et al. 2016).

It is, therefore, this work's goal to further our understanding of the impact metabolic stress has on fetal development, particularly in regard to allometric growth, thus allowing us to develop better strategies to manage this condition and prevent its burdens from afflicting calf productivity and welfare in the future.

3. Bibliographic revision

3.1. Fetal growth

Initial development of major fetal organs occurs as early as the first month of gestation. At month number four most of the gross organ characteristics are defined and similar to those of the neonate and during the remaining five months, the cellular development of major organs is completed (Guyton and Hall 2011). The majority of fetal growth occurs in the final two to three months of gestation, when the fetus grows at its fastest rate. For dairy cattle, around 60% of fetal growth occurs during this time (Bauman and Currie 1980; Andrews et al. 2004).

One of the cornerstones of fetal growth is nutrient availability. Several other factors play a role in prenatal development but access to glucose, lactate and amino acids, the main substrates used by the fetus, is an absolute must (Pere 2003; Guyton and Hall 2011). The supply of these nutrients to the fetus is conditioned by other factors: maternal size and nutrition, which represent the quantity and quality of the available nutrients, and the placenta, responsible for all fetal-maternal exchanges and, consequently, the effectiveness of nutrient delivery (Bauer et al. 1998; Bell and Ehrhardt 2002).

Placental function is, therefore, of the utmost importance for fetal growth and development. It is responsible for the delivery of nutrients and oxygen to the fetus and the removal of waste from fetal metabolism, as well as endocrine regulation of maternal-fetal exchanges. (Bauer et al. 1998; Bell and Ehrhardt 2002).

3.1.1. Endocrine factors in prenatal growth

Several hormones are heavily entwined with the regulation of fetal growth. Based on the works of Fowden (1995), Bauer et al. 1998, Hafez B and Hafez ESE (2000) and Lawrence and Fowler (2002) the influence these hormones have in the growth, maturation and differentiation of fetal tissues has been reviewed.

Growth hormone, despite being essential for postnatal growth and being capable of promoting it prenatally, usually has no direct influence in fetal growth. In contrast, thyroid hormones and glucocorticoids play an important role in the maturation of fetal tissues: thyroxine, promotes oxygen intake to fetal tissues, important for both their growth and maturation, while cortisol heavily influences tissue maturation and differentiation, in addition to helping regulate fetal IGFs concentrations.

Insulin is directly associated with the size and weight of the newborn and enables both fetal and placental growth by promoting glucose uptake. Additionally, it helps regulate insulin like growth factor 1 (IGF-1) concentrations in fetal circulation. In turn, insulin like growth factor 2 (IGF-

2) is regulated more directly by fetal glucose levels and mediates fetal growth based on glucose availability as well.

Despite having a lower expression than IGF2 in fetal tissues, IGF1 seems to play a bigger role in prenatal growth. IGF1 concentrations are directly connected to fetal and placental weight and size at birth (Agrogiannis et al. 2014). However, this influence might not be uniform for all fetal tissues since a study by Lok et al. (1996) showed that IGF-1 supplementation to sheep in late gestation increased the weight of some major fetal organs in detriment of others.

3.1.2. Intrauterine growth retardation

Due to a recurrence in human pregnancies, several experimental models have been performed in sheep to study what is now referred to as intrauterine growth retardation (IUGR). Essentially, in IUGR, prenatal growth is stunted, and birth weights are lowered, due to an inadequate nutrient supply to the developing fetus. The cause of this condition can usually be attributed to one of two factors: placental insufficiency or nutritional anomalies (Wallace et al. 2005; Morrison 2008).

The mechanisms at work in IUGR have been investigated by several authors. Osgerby et al. (2002) linked IUGR in malnourished sheep with lower fetal concentrations of glucose, insulin and IGF1. Limesand et al. (2006) proved that fetal insulin secretion was impaired due to deficits in either insulin storage or production, thereby explaining the fetal hypoinsulinemia. Hay (2006) observed that, despite the fetal hypoglycemia, glucose usage by fetal tissues remained relatively equal and, in turn, Limesand et al. (2007) determined that an increased insulin sensitivity and a precocious start in fetal gluconeogenesis were the most likely explanation for this. A similar range of effects to fetal metabolism could also be observed when IUGR was studied in cattle (Long et al. 2009).

What is particularly interesting is that some studies (Osgerby et al. 2002; Wallace et al. 2005; Morrison 2008; Gao et al. 2009; Long et al. 2009; Yates et al. 2011) noticed that certain organs, frequently the brain, which are more crucial for survival, were partially “spared” from the weight reduction that affected other tissues, resulting in a disproportionate or asymmetrical growth retardation. Yates et al. (2011) and Morrison (2008) linked this phenomenon to the differences between insulin dependent and independent tissues (skeletal muscle, liver, heart and adipose tissue vs brain and nervous tissue, respectively), that allow the brain to avoid the more detrimental effects of glucose scarcity, and shifts in blood perfusion, that favoured blood supply to the brain, heart and adrenal glands.

3.2. Transition period and negative energy balance

When pregnant dairy cows approach delivery time, they go through one of the most stressful periods of their life. In the 3 weeks before and after calving, also known as the transition period, dairy cattle go from a gestational non lactating state to a sudden and exponential increase in milk production. This transition represents a very vulnerable time in a dairy cow's life. One of its defining features, especially in high yield dairy cows, is a negative energy balance (Goff and Horst 1997; Drackley 1999; Herdt 2000).

In the final weeks of gestation, cow's dry matter intake (DMI) progressively diminishes, and only after calving does it begin to rise back to normal. Simultaneously, the start of lactation at calving marks a sudden increase in the cow's energetic needs. DMI increases after calving in an attempt to keep up with these needs, however, it is too slow to keep up with such an abrupt and swift increment. Moreover, years of genetic selection have led the dairy cow to produce far greater quantities of milk than physiologically required for its offspring, further exacerbating this situation (Grummer et al. 2004; Gordon 2004).

This discrepancy between the nutrient uptake and the rapidly rising energy requirements is what leads to NEB and a complex series of metabolic adaptation mechanisms are triggered in order to manage it (Herdt 2000).

3.2.1. Ruminant glucose metabolism

All cells can use glucose as an energy substrate, however there are some cells and tissues that meet their energetic needs exclusively through it: brain, mammary and fetal tissues are a prime example of this (Aschenbach et al. 2010). There are three major processes that regulate this carbohydrate's metabolism: gluconeogenesis, glycogenolysis and glycogenesis. Gluconeogenesis consists in the production of glucose molecules by using non carbohydrate precursors (most commonly lactate, glycerol, and amino acids). When there is an abundance of glucose precursors and the production of glucose surpasses the energetic needs of the animal, excess glucose can then be turned into glycogen (glycogenesis) and stored in the liver. Glycogenolysis, in turn, is the breakdown of the glycogen molecules back into glucose, in situations of scarcity.

Due to the peculiarities of their digestive system, ruminant glucose metabolism is slightly different from other species. Due to ruminal fermentation, the carbohydrates ruminants ingest are reduced to short chain fatty acids. Since essentially no glucose in their diet can be absorbed, ruminants are then forced to meet their glucose demands by resynthesizing it, through gluconeogenesis. However, ruminants do not use the same glucose precursors as monogastric

mammals. The amount of lactate that ruminants get through their diet is negligible so instead they use the volatile fatty acids (VFA) that are produced by microbial carbohydrate fermentation. The most abundant VFA produced in the rumen are propionate, butyrate and acetate, yet, out of these three, only propionate can be used for glucose synthesis, making it the main precursor for ruminant gluconeogenesis (Young 1977; Aschenbach et al. 2010).

3.2.2. NEB adaptations in glucose metabolism

Glucose supply is necessary to power not only the cows own metabolic needs, but fetal and lactational needs as well. However, during the transition period, the majority of available glucose is diverted towards the mammary gland and the uterus. In order to keep up with the severity of these demands, the dairy cow is forced to increase the rates of both gluconeogenesis and glycogenolysis, to increase the amount of glucose available (Bell and Bauman 1997; Herdt 2000).

Being dependent on the availability of propionate, glycerol and amino acids, ruminant gluconeogenesis during this time presents several issues. While propionate can be obtained through the diet, but even that is limited by the lowered DMI, the main sources of glycerol and amino acids are the adipose and muscle tissue, respectively. In order for glucose synthesis to be a sustainable energy source, extensive fat and muscle breakdown would be necessary, an unacceptable long-term solution (Young 1977; Aschenbach et al. 2010). On the other hand, glycogenolysis is dependent on the glycogen reserves of the body which, unfortunately, are nowhere near enough to keep up with its demands (Herdt 2000). This does not mean, however, that these two mechanisms do not play an important role in the management of NEB just that, by themselves, they would not be able to sustain all the energetic needs of lactation, gestation and maintenance. Therefore, in order to spare as much glucose as possible for milk production and fetal development, alternative energy sources are used to fuel the needs of other tissues.

3.2.3. NEB adaptations in lipid metabolism

Fat is the greatest energy reserve in the body. By mobilizing the energy stored in the adipocytes dairy cattle manages to adequately respond to the energetic demands of the transition period (Herdt 2000). The adipose tissue stores energy in the form of triglycerides (three fatty acids bound to a glycerol molecule through an ester bond). In normal conditions, there is a balance between the production and breakdown of triglycerides however, when NEB occurs, it tips in favour of lipolysis. By cleaving the ester bond and splitting the triglyceride back into its base components, an increase in circulating non esterified fatty acids (NEFAs) and glycerol occurs (van

der Kolk et al. 2017). This mobilization and increase in blood levels of NEFAs is considered to be a hallmark of the transition period (Bell 1995). Bergman (1971) described three possible pathways by which NEFAs could be metabolized: complete oxidation, partial oxidation and re-esterification.

Many peripheral tissues, skeletal muscle being of particular note, can use NEFAs as an energy source when glucose is in short supply (Bell 1995; Drackley 1999). The oxidation of NEFAs, also referred to as β-oxidation, is a necessary step for energy production using fatty acids. As reviewed by Adewuyi et al. (2005) and van der Kolk et al. (2017), β-oxidation is the breakdown of NEFAs into smaller chain fatty acids that occurs mainly in the mitochondrial matrix. Their reduction down to acetyl-coA allows them to then be received by oxaloacetate (OAA) to enter the Krebs cycle. When glucose is used as fuel, a new OAA molecule is formed to replace the previous one but since the energy source in this case is a fatty acid, this step does not occur. Since glucose availability is scarce during the transition period, little to no glucose can be spared for OAA renewal. Therefore, this process becomes reliant on the quantity of OAA already available. The use of fatty acids to power the Krebs cycle is what Bergman (1971) referred to as complete oxidation. When this is no longer an option, circulating NEFAs are seized by the liver to be metabolized in one of the other two pathways.

Partial oxidation is what occurs when acetyl-coA can no longer enter the Krebs cycle. Instead, it is converted to acetoacetyl-coA and used to synthesize ketone bodies (ketogenesis). The main ketone bodies are acetoacetate, B-hydroxybutyrate (BHB) and acetone. Although not exclusively, they are mainly synthesized in the liver and can then be used by many extrahepatic tissues, to meet their energetic needs (Zarrin et al. 2017). The final pathway, re-esterification, is a hepatic process that as the name implies, creates a new ester bond, turning the NEFAs back into a triglyceride. They can then either be exported from the liver as very low density lipoproteins or be stored as liver lipids (White 2015).

It is worth mentioning, however, that if this production of triglycerides surpasses the liver's capacity to export them, it can lead to their accumulation in hepatic tissue, causing the metabolic disease known as fatty liver. Likewise, there is also a limit to the amount of ketone bodies the organism can metabolize, after which they may be found in the blood, urine and milk, causing the condition known as ketosis. Extensive fat mobilization and high concentrations of circulating NEFAs are, therefore, a risk factor for the incidence of both these diseases. (Herdt 2000).

3.2.4. Feedback mechanisms between glucose, NEFAs and ketone bodies

The mechanisms that regulate NEFA and ketone body metabolism are heavily entwined with an animal's glucose status (Herdt 2000). As mentioned above the complete oxidation of

NEFAs is dependent on OAA availability. Since OAA is mostly reliant on glucose for renewal, this effectively allows glucose levels to limit NEFA usage by extrahepatic tissues, therefore influencing NEFA blood concentrations and their redirection towards ketogenesis (White 2015). An additional way by which glucose affects fatty acid metabolism is by regulating their access to the mitochondria. NEFA oxidation occurs almost exclusively inside the mitochondria, and their entry into this organelle is mediated by the enzyme carnitine palmityl transferase I (CPT I). During NEB, when glucose is scarce, NEFA can enter the mitochondria unhindered however, in an euglycemic cow, CPT I function is inhibited by malonyl-coA, stopping their entrance. This effectively obstructs NEFA oxidation, forcing them to instead be redirected towards re-esterification (Holtenius P and Holtenius K 1996; van der Kolk et al. 2017).

The glucose-insulin axis also plays a role in lipid metabolism. The state of hypoglycemia, that dairy cattle find themselves in during the transition period, naturally leads to a concomitant decrease in insulin release. Insulin, in turn, has a direct influence on the synthesis and breakdown of triglycerides in the adipocytes – it stimulates lipogenesis and inhibits lipolysis. The shift towards lipid breakdown that occurs during NEB is, therefore, most likely explained by this hypoinsulinemia (Bell 1995; Herdt 2000).

Taking the outcome of these mechanisms into account, we can see that they all have a clear physiological purpose. In the absence of glucose, the metabolism of alternative fuels is promoted to replace it. In turn, when glucose is abundant, there is no need to rely on alternate, less safe, energy sources, and therefore their metabolism is supressed. That said, both NEFAs and ketone bodies have additional roles besides glucose sparring.

Clearly NEFA concentration has a direct relation to ketogenesis, however ketone bodies also have a feedback function towards NEFAs. When insulin activity is decreased, ketone bodies can inhibit lipolysis, thereby preventing fat mobilization, NEFAS and themselves from rising uncontrollably (Holtenius P and Holtenius K 1996). In addition, If allowed to grow unchecked, both NEFAs and ketone bodies can cause grievous harm to glucose metabolism. Fatty liver has been shown to impair gluconeogenesis (Adewuyi et al. 2005). Likewise, high concentrations of BHB have also been linked to decreased rates of glucose synthesis, most likely due to their suppression of protein breakdown, that restricts access to the amino acids needed for the process (Zarrin et al. 2017). In both cases the curbing of glucose production, in an animal already suffering from a shortage of it, is bound to have a highly detrimental effect on that animal's energetic balance.

In summation, the adaptation mechanisms that occurs during the transition period are a delicate balance between glucose, NEFAs and ketone bodies. Although in most cases some

degree of NEB is unavoidable, its escalation into a more serious condition can be prevented by not further stressing the transition cow's metabolism. (Herdt 2000).

3.3. Metabolic stress

In humans, metabolic stress has been described as an "*imbalance in the physiological homeostasis of an organism as a consequence of aberrant nutrient utilisation*" (Sordillo and Mavangira 2014, p. 1205). Likewise, Abuelo et al. (2015, p. 1004) referred to dairy cattle metabolic stress as a "*hypermetabolic, catabolic response to an imbalance in physiological homeostasis*" that is typically related to transition period hypoglycemia. By both definitions' standards, insufficiencies in homeostatic regulation seem to be a key aspect of metabolic stress. This can be largely explained by realising that the metabolic adaptations that lead to this condition are not regulated by homeostasis, but by homeorhesis instead. Bauman and Currie (1980) reviewed the concepts of these two regulatory mechanisms and how they differed from each other. While homeostasis strives to maintain a biological equilibrium through a series of physiological processes, homeorhesis pushes towards a specific physiological goal with combined efforts throughout the metabolism. Taking this into account, it seems clear that the metabolic adaptations that prioritize nutrient supply towards lactation and gestation even in a state of NEB, are homeorhetic mechanisms.

Regardless, there seems to be a consensus that metabolic stress occurs when the adaptation mechanisms are overrun. In fact, Sordillo and Raphael (2013) stated that metabolic stress is not the result of the NEB metabolic adaptations themselves, but the loss of the feedback mechanisms that regulate them. Considering the previous statement, we can surmise that either atypically high energetic requirements or anomalous use of available nutrients or both, should be necessary to overwhelm the adaptation mechanisms of the transition period.

When we look at dairy cattle through that lens, there are certain "risk" groups that stand out as more susceptible to develop metabolic stress: high yield cows, pregnant heifers, gemelar pregnancies, cows with a high or low body condition score (BCS) and heat stressed cows (Costa 2015; Sordillo 2016). Naturally, both high yield dairy cows and gemelar pregnancies, have additional energetic demands associated with lactation and gestation, respectively. Lactating cows, in particular, are more prone, considering that dairy breeds have been progressively selected to maximize production. Gestating heifers as well, are bound by an increment in energetic requirements, since it is standard practice for heifers to be inseminated before they fully mature, and therefore need to match the energy needed to simultaneous promote gestation, and their own growth and development (NRC 2001).

Extreme body condition scores can also be detrimental. BCS is directly related to the total fat content of the animal and, therefore, its energetic reserves. Too low BCS means that the reserves are limited, and a more pronounced NEB will occur. In turn, high BCS mean that, in addition to bigger subcutaneous fat deposits cow's also have a greater accumulation of intra-abdominal fat. This translates into less ruminal space and, coupled with a lower ambulation to search for food, a more pronounced decrease in DMI. Together with the enhanced lipolytic activity of the transition period, this encourages excessive lipid mobilization, with a corresponding increase in NEFA and ketone body concentrations above an acceptable threshold. Thereby justifying the higher incidence of peripartum metabolic diseases in high BCS dairy cows (Bewley and Schutz 2008; Sordillo and Raphael 2013).

Heat stressed cows are a particular case that is further explored later in this paper but, in summation, due to the complex effects heat stress can have on the energetic metabolism it can lead to an aberrant use of available nutrients, therefore, predisposing to metabolic stress (Radostits et al. 2007; Sordillo and Raphael 2013).

Irrespective of how they reach metabolic stress, dairy cattle virtually always present three heavily entwined characteristics: excessive lipid mobilization, dysfunctional inflammatory response and oxidative stress (Sordillo and Mavangira 2014; Abuelo et al. 2015), as will be explained in the next sections.

3.3.1. Excessive lipid mobilization

According to Sordillo and Raphael (2013) an excessive lipid mobilization is defined by a blood NEFA concentration too high to be safely metabolized. The link fatty liver and ketosis have with an increase of circulating NEFAs has already been established yet, in spite of that, not all cows that go through lipid mobilization develop these diseases (Herdt 2000). Although, usually, insulin dependent feedback mechanisms can regulate NEFA concentrations and keep them within acceptable limits, in the transition period this is not the norm. Transition cows are known to develop some degree of insulin resistance in peripheral tissues (De Koster and Opsommer 2013). The exact mechanisms that lead to this resistance are still unclear although there is circumstantial evidence that suggests an increase in tumor necrosis factor α (TNF-α) might be responsible (Ohtsuka et al. 2001), further backed by similarities found in steers (Kushibiki et al., 2001) and other animal studies (Hotamisligil 2009).

Nevertheless, this shift in insulin sensitivity coupled with the already high propensity towards fat mobilization in the transition period means that NEFAs levels can rise unhindered, greatly increasing the cow's risk of succumbing to one of the aforementioned metabolic diseases.

3.3.2. Dysfunctional inflammation

An adequate inflammatory reaction balances the ability to eliminate the pathogen or insult that triggered it, with a prompt return of the involved tissues to normal morphology and function (Sordillo and Raphael 2013). A dysfunction in this process can therefore occur due to either an underactive or an overactive response. A standard acute inflammatory reaction starts with insult detection by epithelial cells and subsequent release of proinflammatory molecules such as nitric oxide, eicosanoids and cytokines, that trigger all other inflammatory phenomenon's (vasodilation, edema, and chemotaxis). If the reaction is hypoactive, the leukocytes response time will be delayed, allowing the proliferation of disease. In turn, a hyperactive inflammatory response can lead to an exaggerated reaction or a chronic inflammation, with potential damaging effects to the inflamed tissue (Guyton and Hall 2011; Sordillo and Mavangira 2014).

3.3.3. Oxidative stress

Oxidative stress is the result of an imbalance between the oxidants and antioxidants in the organism. Both an exacerbation of oxidant production and a depletion of antioxidant supplies can cause it (Sordillo and Raphael 2013; Abuelo et al. 2015). The β-oxidation of NEFAs in peripheral tissues creates various reactive oxygen species (ROS), oxidants, as a by-product. The intense lipid mobilization in the transition period can therefore be linked to an increased production of ROS. However, endogenous supplies of antioxidants remain the same and once spent, oxidative stress occurs (Schonfeld and Wojtczak 2008; Sordillo and Aitken 2009).

In adequate amounts, ROS play a role in several physiological functions, their capacity to optimize the inflammatory reactions in the early stages of disease being of particular note (Sordillo and Raphael 2013; Abuelo et al. 2015). The problem arises when large quantities of ROS build up, which can cause extensive tissue damage (by oxidizing cellular components like lipids, proteins and DNA) and destabilize both inflammatory and immune responses (Sordillo and Aitken 2009; Abuelo et al. 2015).

3.3.4. Interrelations in the metabolic triad

There are several ways by which the three facets of metabolic stress influence each other, for instance, Contreras and Sordillo (2011) linked fat mobilization, and its associated rise in NEFA concentrations, with increased inflammation, abnormal immune cell functions and increased risk of metabolic and infectious diseases in dairy cows.

The mobilization of fatty acids that occurs due to NEB, leads to changes not just in quantity but also in composition of circulating NEFAs. In turn, the lipid composition of immune cells, like

leukocytes and endothelial cells, mirrors the composition of the lipid fraction in plasma (Contreras et al. 2010). The fatty acids that are assimilated into these cells, have the capacity to influence the inflammatory response. In leukocytes, the saturated fatty acids, like palmitate, stearate, and oleate, have been shown to have pro-inflammatory aptitudes, in contrast, polyunsaturated fatty acids (PUFAs), seem to favour a more anti-inflammatory approach (Sordillo and Raphael 2013).

During the transition period, the proportions of palmitic acid in plasma NEFA are increased, while PUFA quantities are diminished. This shift towards saturated fatty acids may partially explain the heightened inflammation exhibited by the transition cow (Contreras et al. 2010). Building on this concept, further attempts at linking increased fat mobilization with an intensified inflammatory response have been made. Eicosanoids, inflammatory signalling molecules, can be synthesised by two types of PUFAs, omega-3 or omega-6. Omega-6 derived eicosanoids (prostaglandins, leukotrienes, and thromboxanes) all have proinflammatory functions. In turn, eicosanoids synthesised by omega-3 fatty acids (protectins and resolvins) have been shown to promote anti-inflammation (Serhan 2009). Two studies (Contreras, Mattmiller et al. 2012; Contreras, Raphael et al. 2012) based on the exposure of bovine endothelial cells to omega-3 fatty acids and NEFAs from a transition cow, respectively, led to the conclusion that the increased inflammatory response during the transition period could be explained by a dominant presence of omega 6 PUFAs in the composition of the transition cow's circulating NEFAs.

It seems then, that an excessive mobilization of lipids can have an aggravating effect on both other aspects of metabolic stress. In the inflammatory response, through changes in plasma NEFA quantity and composition, and in oxidative stress, through increased β-oxidation also brought on by changes in NEFA concentration.

In a similar way, both enhanced inflammation and oxidative stress, seem to have a corresponding exacerbating effect on lipid mobilization by increasing the rates of lipolysis (Sordillo and Raphael 2013; Abuelo et al. 2015). Additionally, these two factors can also directly influence each other. The role ROS play in immune function is responsible, among others, for the increased expression of several proinflammatory factors, such as cytokines and eicosanoids. In turn, inflammatory processes like phagocytosis increase ROS generation, meaning both inflammation and oxidative stress reciprocally intensify their effects (Sordillo and Raphael 2013; Abuelo et al. 2015). This synergism has been linked to a higher incidence of infectious diseases like mastitis in the transition cow (Turk et al. 2017).

In summation, these conditions influence one another, potentiating each other's effects and creating a vicious cycle that further exacerbates the transition cow's metabolic stress (Sordillo and Mavangira 2014).

3.3.5 Metabolic stress impact on the fetus

Although the detrimental effects of metabolic stress to the dam have been widely studied, little is known on if and how it can affect fetal development. In fact, only one study (Ling et al. 2018) could be found that actively researched the impact of metabolic stress in the prenatal calf. In this study, metabolic status of the cows was assessed through serum concentration of NEFA and haptoglobin and oxidant status. Increases in these biomarkers, respectively coincided with increments in cow lipid mobilization, inflammation and oxidative stress. The study was able to link marked increases in lipid mobilization, inflammation or oxidant stress in the cow to lower birth weights, greater ROS concentrations and shifts in the inflammatory response of the neonate. Furthermore, some of these effects could still be shown at one month of age. While the mechanisms behind these results are still unknown, conclusive evidence was found that late gestation metabolic stress can impact fetal growth and metabolism, and possibly have carryover effects to the calf's adult life.

Sadly, further studies on this relationship are still lacking. However, several have been published on the connection between heat stress, which relates to enhanced oxidative stress and inflammatory responses in transition cows (Sordillo and Raphael 2013), and fetal development during late gestation.

3.4. Heat stress

Cattle, like all mammals, are an homeothermic animal, meaning that they regulate their body temperatures based on the variation of environmental temperatures. Homeotherms have an optimal temperature range, where their performance is at its best and no excess energy is expended to thermoregulate the body, known as the thermoneutral zone or the thermal comfort zone. However, this is also heavily influenced by relative humidity, with higher percentages being linked to decreased performance outcomes (Kadzere et al. 2002; West 2003; Andrews et al. 2004; Reece and Rowe, 2017). For lactating dairy cattle, the upper limit of this range at 35 to 50% relative humidity as been determined to be around 25 ºC (Berman et al. 1985). Heat stress occurs when environmental temperatures surpass this limit, causing animals to employ a series of behavioural and/or physiological mechanisms in order to regulate their own body temperature.

Cattle deals with heat stress by lowering heat production through behavioural changes like decreasing their activity levels and their DMI. They can also promote heat loss by sweating and panting or by increasing peripheral blood flow to raise heat dissipation. Normally, about 75% of heat loss happens this way (Kadzere et al. 2002; Andrews et al. 2004; Reece and Rowe 2017).

Additionally, many practices nowadays are employed to assist in preventing heat stress in dairy farms such as cooling fans, showers and ample shaded areas (West 2003; Andrews et al. 2004).

3.4.1. Changes in performance and metabolism during heat stress

The detrimental effects heat stress can have in lactating dairy cows are various: decreased DMI, lower milk yield, metabolic changes and higher incidence of diseases (Kadzere et al. 2002; West 2003). Looking further into the metabolic changes, a few studies (Wheelock et al. 2010; Baumgard and Rhoads 2012; Tao and Dahl 2013) have shown that, despite a reduction in DMI, lactating heat stressed cows will hinder fat mobilization and favour glucose use in peripheral tissues. This comes at the price of diverting less glucose towards the mammary gland, possibly explaining the decrease in milk production that lactating heat stressed cows present. An increase in insulinemia seems to explain this by inhibiting lipolysis. However, the reason for the elevated insulin value remains unknown. Additionally, this glucose preference is aided by a higher rate of gluconeogenesis and glycogenolysis, and strangely enough, an increase in muscle breakdown, possibly to provide amino acids for glucose synthesis in the liver.

During the transition period the same adaptations do not occur in late gestation dry cows. For starters, when compared to lactating heat stressed cows, heat stressed dry cows show an increase in circulating NEFAs, coupled with an increase in blood BHB and lower levels of glucose and insulin. Most evidence points towards glucose supply being diverted to the uterus for the final stage of fetal development and, therefore, alternative energy sources are needed for the dam's peripheral tissues. Nevertheless, while the degree of fat mobilization of heat stressed cows in late gestation is greater than in heat stressed lactating cows, it is still lower than what occurs in dry cows during thermoneutral conditions. Inversely, when comparing proteolysis in heat stressed cows, its incidence during the dry period is lower than during lactation, but even lower still in thermoneutral dry cows (Do Amaral et al. 2009; Tao and Dahl 2013; Lamp et al. 2015; Koch et al. 2016).

Besides metabolic shifts, heat stress during the dry period causes dairy cattle to present a diminished DMI (same as with lactating cows), a reduced milk yield in the following lactation and birth calves with smaller birth weights (Collier et al. 1982; Tao and Dahl 2013). Additional studies have also been conducted on the influence of late gestation heat stress to the unborn calf and the carryover effects it can have postnatally. Monteiro et al. (2016) reviewed how prenatal heat stress influences calf performance postnatally: higher rates of morbidity and mortality, lower milk output during first lactation, inferior reproductive performance, and lower body weights during their first year.

The mechanisms behind smaller calf birth weight have also been researched. Cattle that suffer heat stress during gestation have shown reduced blood flow to the uterus (Roman-Ponce et al. 1978; Reynolds et al. 1985) and decreased placental weight and hormone production (Collier et al. 1982) Heat stressed cows also have a shorter gestation time of up to 4 days (Tao et al. 2012). Due to the importance both gestation length and placental function have in fetal growth, an argument can be made that these are responsible for the lower birth weight of calves that suffer prenatal heat stress (Reynolds et al. 2006; Tao and Dahl 2013).

4. Objective

The purpose of this study was to determine if maternal metabolic stress can cause prenatal calf to prioritize the development and growth of major organs such as heart, lungs and brain, determined indirectly through the measurement of the thorax and skull, over the growth of less vital organs such as the limbs.

Additionally, we proposed to test if by performing the study in different countries, with distinct environment air temperatures, any variances in outcome could be attributed to heat stress.

5. Material & methods

5.1. Farm characterization

This study was performed in a dairy farm 35 km north of Lisbon, Portugal.

The farm herd is around 800 high yield lactating Holstein Friesian cows that are kept year-round in several free-stall barns with sand bedding and milked three times a day. In the last two months of gestation, it is standard procedure to dry off the cows and move them from the general population to a dry-cow barn and then to a maternity area, around 15 days before calving is due. The maternity area is on a one open sided brick barn, with straw bedding.

Additionally, this same study was performed in two dairy farms in Ghent, Belgium, BE1 and BE2. Farm BE1's herd is around 1500 high yield Holstein Friesian cows housed in a cubicle barn and milked 3 times a day. They spent their final month of gestation in a maternity area with straw bedding. Farm BE2's herd is far smaller, only around 150 Holstein Friesian cows that are milked twice daily, and housed year round in deep litter boxes with sand bedding.

5.2. Sample characterization

All cows in the Portugal farm that gave birth during the months of July and August of 2017, and their respective calves, were used in the study. During those two months, exactly 96 cows gave birth to a total of 100 calves. Four of these births resulted in twins.

Regarding farms BE1 and BE2, the respective data from 62 and 9 calves, born to an equal number of cows, was used in the study. The data collected from both these farms was obtained in two separate occasions: season 1, measured at the end of February 2017 (22/02/2017-25/02/2017), and season 2, measured at the end of March 2017 (30/03/2017). These measurements were used for comparison with the Portuguese data, in hopes of highlighting differences based on season and average air temperature (Table 3).

Table 3. Discrepancies in season and average temperature between farms.

	Season 1 End of February 2017 (4.5 ºC)	Season 2 End of March 2017 (5.3 ºC)	Season 3 July & August 2017 (25 ºC)
Farm BE1	41 calves	21 calves	-
Farm BE2	8 calves	1 calf	-
Farm PT	-	-	100 calves

5.3. Study characterization

The prenatal growth and development of the newborn calves' limbs and organs was quantified by taking several bone measurements postnatally. Every calf was measured before one week of age, so that their results would not be unduly influenced by postnatal growth.

The gender of the newborn calves, as well as the dates of their birth and when they were measured were also registered.

As for the mothers, metabolic stress was determined indirectly, using data collected from the farm's production logs. Based on the higher risk groups associated with metabolic stress, milk production for the reproductive year (M305d) and the number of births for each individual cow (parity) were chosen as indicators. Respectively, they underlined higher energy requirements related to lactation and with heifer gestation.

Twin births and date of conception were also recorded to, respectively, keep track of changes based on gemelar pregnancies and gestation length.

5.3.1. Measuring protocol

The measurements were performed according to the following protocol, with a measuring tape and a calliper:

- Height at withers (WH "withers height"), the height of the calf, measured with a calliper at the highest point of the withers, on the standing calf (Figure 1).
- Chest circumference (HG "heart girth"), the circumference of the chest, measured with a tape measure on the standing calf, just caudal of the elbow (Figure 2).
- Diagonal length (DL), the length of the calf, measured on the standing calf with a calliper, between the most cranially point of the shoulder, the tuberculum majus humeri, and the most caudal point of the tuber ischiadicum (Figure 3).
- Shoulder width (SW), the width of the calf at the shoulders, measured on the standing calf with a calliper, at the widest point of the shoulder region (Figure 4).
- Hip width (HW), the width of the back of the calf, measured with a calliper on the standing calf, at the widest point of the pelvic region (Figure 5).
- Head circumference (HC), the circumference of the head of the calf, measured on the standing calf, with a tape measure just rostral of the ears (Figure 6).
- Head diameter (HD), the diameter of the head of the calf, measured on the standing calf with a calliper, between the lateral aspects of the bony orbit (lateral of the eyes) (Figure 7).

- Metatarsus length (ML), the length of the left metatarsus of the calf, measured on the lying calf with a calliper. The fetlock is bent 90° and the length is measured between the cranial aspect of the fetlock and the most caudal point of the hock (tuber calcaneus) (Figure 8).
- Forearm length (FL), the length of the left forearm of the calf, measured on the lying calf with a calliper. The carpus is bent 90° and the length is measured between the cranial aspect of the carpus and the most caudal point of the elbow (dorso-caudal aspect of the ulna) (Figure 9).

Figure 1. Measurement of height at withers. Picture provided by Prof. Dr. Geert Opsomer.

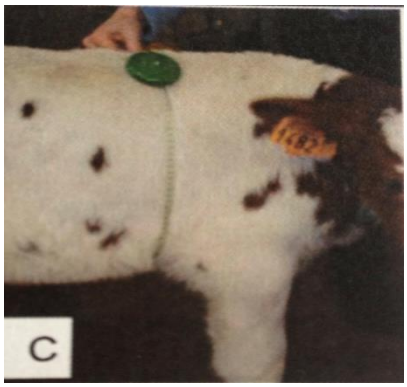

Figure 2. Measurement of chest circumference. Picture provided by Prof. Dr. Geert Opsomer.

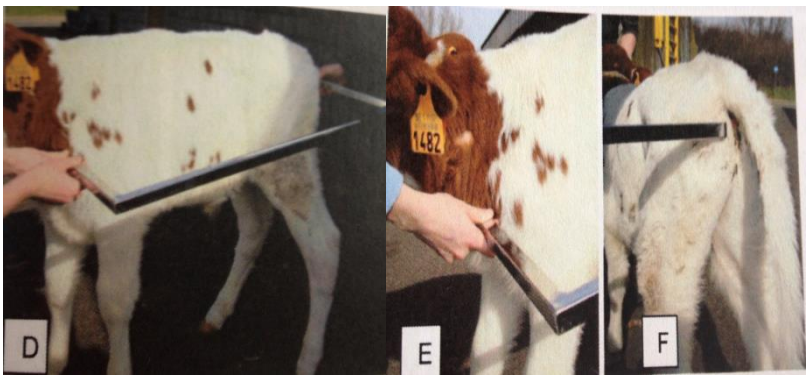

Figure 3. Measurement of diagonal length with detail of the cranial and caudal positioning of the calliper. Picture provided by Prof. Dr. Geert Opsomer.

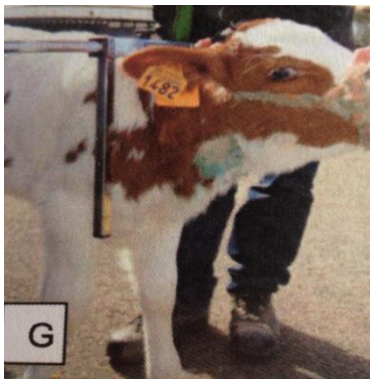

Figure 4. Measurement of shoulder width. Picture provided by Prof. Dr. Geert Opsomer.

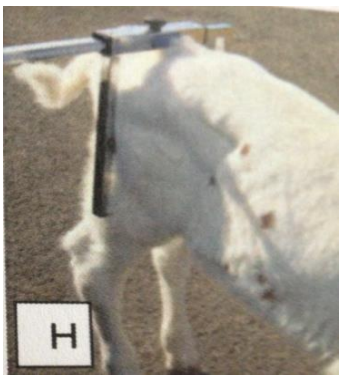

Figure 5. Measurement of hip width. Picture provided by Prof. Dr. Geert Opsomer.

Figure 6. Measurement of head circumference. Picture provided by Prof. Dr. Geert Opsomer.

Figure 7. Measurement of head diameter. Picture provided by Prof. Dr. Geert Opsomer.

Figure 8. Measurement of metatarsus length. Picture provided by Prof. Dr. Geert Opsomer.

Figure 9. Measurement of forearm length. Picture provided by Prof. Dr. Geert Opsomer.

The measuring protocol was developed by UGent's – Faculty of Veterinary Medicine, Department of Obstetrics, reproduction and herd health in association with their Department of Morphology, in the most standardized and systematic approach possible, taking care to always do the ML and FL measurements from the left limbs and all other measurements on the fully standing calf. The protocol was performed by different people in the Portuguese and the Belgian farms (the author and another student from UGent, respectively) however prior to those instances we both practiced the protocol together in order to diminish any potential idiosyncrasies we may have had in the measuring technique.

5.4. Statistical analysis

The Portuguese data, as well as the Belgian data were both analysed separately and as a combined set, by the R statistics software (version 3.4.1).

The first step was the creation of new variables to quantify the allometric growth of the vital organs (brain, heart, lungs), represented by the cranium and thorax measurements, against the growth of the long bones, represented by the forelimb and hindlimb measurements. With that in mind, five measurements (HC, HD, HG, ML, FL) were selected to calculate six ratios: HC/ML, HC/FL, HD/ML, HD/FL, HG/ML, HG/FL.

Besides the ratios, another variable (Primi_Multi) was created by splitting parity between primiparous (parity equal to 1) and multiparous (parity greater than 1) animals, in order to determine differences between heifers and lactating cows.

5.4.1. Portuguese data

Starting with the Portuguese data, after an initial outlier search and treatment, the distribution of each ratio was determined with a standard Shapiro Wilk test.

Linear regression models were then executed for each measurement (HC, HD, HG, ML, FL, DL, WH, SW, HW) and ratio (HC/ML, HC/FL, HD/ML, HD/FL, HG/ML, HG/FL) with gender, parity, primi_multi and M305d.

5.4.2. Belgian data

The Belgian data underwent the same process as the Portuguese one: first outlier treatment and normality checks with the Shapiro Wilk test, followed by regression models between each measurement and ratio with gender, parity and primi_multi. Two additional variables, average temperature (temp_av) and season, were used in the Belgian data as independent variables.

5.4.3. Combined data

In similar fashion, outliers were treated, and a Shapiro Wilk test was used to detect normality as the first steps of the combined data analysis.

Analysis of variance (ANOVA) was used to test differences in variation between farms for HC/ML.

Regression models were applied to test if said variation could be linked to gender, parity, primi_multi, M305d, season or average temperature.

Another ANOVA was performed to test differences in variation between seasons for HC/ML and regression models were also used to see how it related to the other variables (gender, parity, primi_multi, M305d and temperature average).

Additionally, a random effect analysis was performed, with farm standing as the random effect, for each ratio with parity, primi_multi and season.

Finally, simple regression models were used for each ratio (HC/ML, HC/FL, HD/ML, HD/FL, HG/ML, HG/FL) and independent variable (gender, parity, primi_multi, M305d, season and temp_av) pairs.

6. Results

The complete register of measurements and all data collected from the study in Portugal can be found in Appendix I.

6.1. Portuguese calves

Head diameter, withers height and shoulder width, all showed a significant correlation to gender (Figures 10, 11 and 12). The values of these three measurements were significantly higher in male calves than in female calves ($p<0.05$).

Figure 10. Differences in head diameter between male and female portuguese calves.

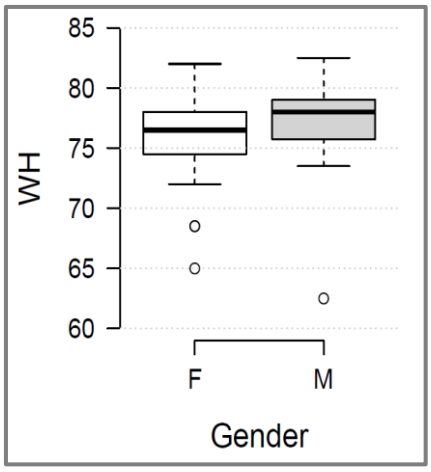

Figure 11. Differences in withers height between male and female portuguese calves.

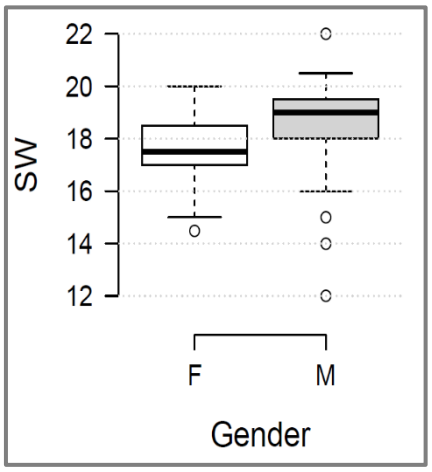

Figure 12. Differences in shoulder width between male and female portuguese calves.

Only one measurement showed a significant correlation to parity. Head diameter was significantly higher in parity 6 calves ($p<0.05$) but also trended higher in parity 4 calves (Figure 13). Heart girth trended higher in both parity 4 and parity 6 calves (Figure 14).

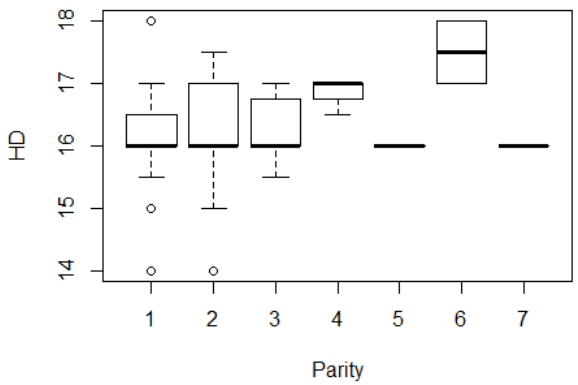

Figure 13. Differences in portuguese calf head diameter based on parity.

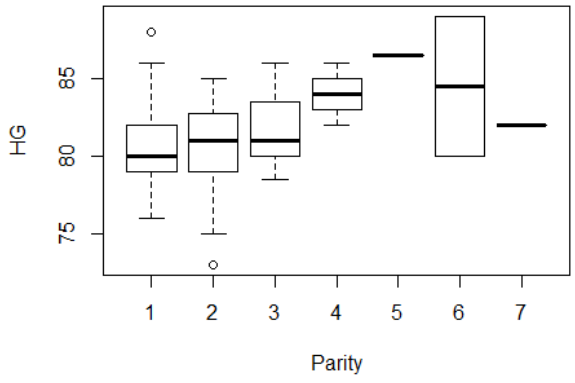

Figure 14. Differences in portuguese calf heart girth based on parity.

Although there were four cases of gemelar pregnancies present they were not used, since their sample size was too small to give any statistically significant results.

No statistically significant results were found between the measurements with primi_multi or M305d, nor between any ratio with any other variable.

6.2. Belgian calves

Statistically significant differences ($p<0.05$) in ratios were linked to parity, primi_multi, season and temp_avg (Table 4). HD/ML, HD/FL, HG/ML and HG/FL were all significantly higher after the first parity (Figures 15 and 16) and, consequently, calves from multiparous cows also had significantly higher values for the same ratios.

In regard to season, calves from season 1 had significantly lower values in all ratios than calves from season 2, as can be evidenced by Figures 17 and 18. Subsequently the ratios values for temp_avg 5.3 ºC also had significantly higher values than those from 4.5 ºC temp_avg.

Table 4. Summary of significant regression model results for ratios in BE_data.

	Gender	Parity	Primi_multi	Season	Temp_avg
HC/ML	-	-	-	P<0.05	P<0.05
HC/FL	-	-	-	P<0.05	P<0.05
HD/ML	-	Parity 2 P<0.05	P<0.05	P<0.05	P<0.05
HD/FL	-	Parity 2 P<0.05	P<0.05	P<0.05	P<0.05
HG/ML	-	Parity 2 & 4 P<0.05	P<0.05	P<0.05	P<0.05
HG/FL	-	Parity 3 & 4 P<0.05	P<0.05	P<0.05	P<0.05

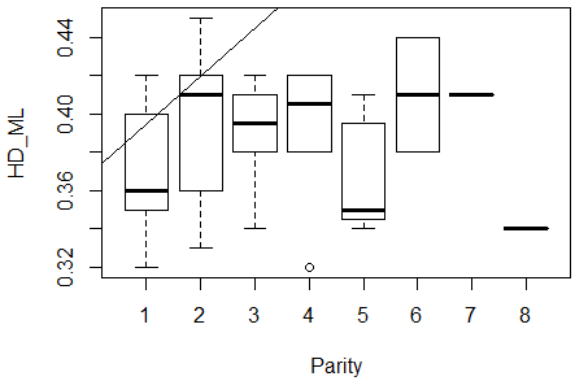

Figure 15. Differences in belgian calf HD/ML based on parity.

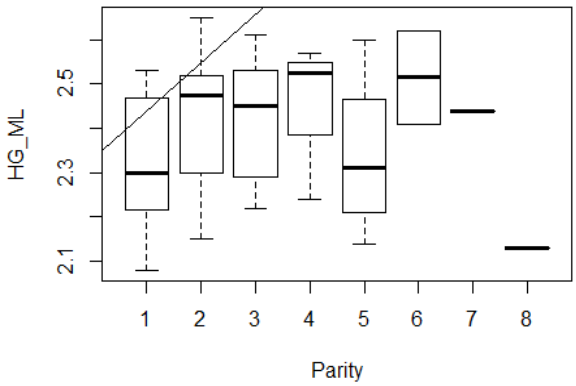

Figure 16. Differences in belgian calf HG/ML based on parity.

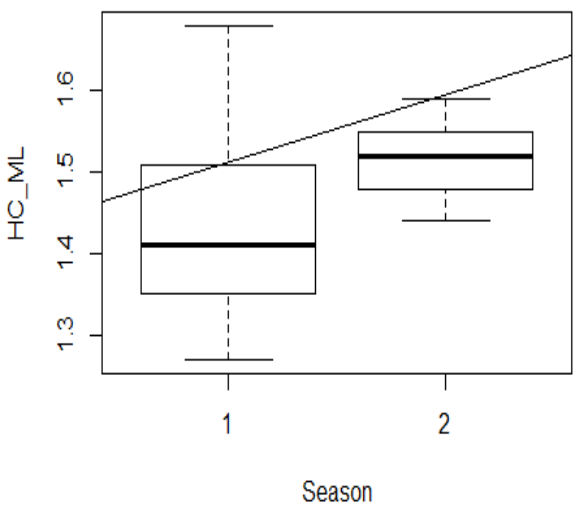

Figure 17. Differences in belgian calf HC/ML based on season.

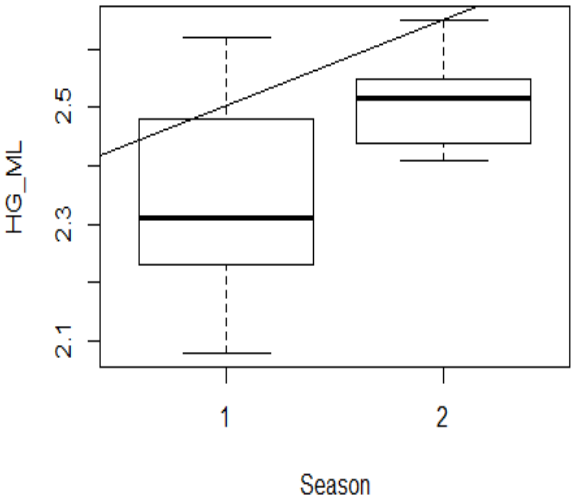

Figure 18. Differences in belgian calf HG/ML based on season.

6.3. Portuguese and Belgian calves

The variance analysis of HD_ML between farms showed statistically significant results ($p<0.05$), which are emphasized on Figure 19. This variation could only be significantly connected ($p<0.05$) to another variable, season. There were no statistically significant results that associated HC_ML farm variation to gender, parity, primi_multi, M305d or average temperature.

The variance analysis of HC_ML also showed statistically significant results ($p<0.05$) between seasons. This variation could not be significantly linked to any other variable.

The random effect analysis did not reveal any new findings: in the absence of farm, season remained the only significant variable.

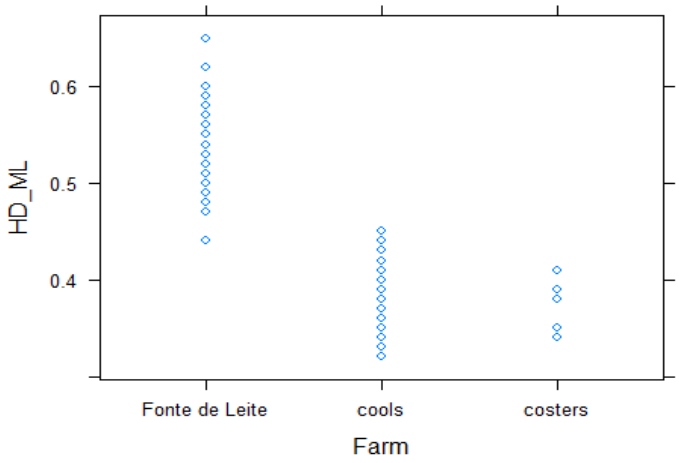

Figure 19. Differences in HC/ML values based on farm.

The simple regression models showed statistically significant results ($p<0.05$) for every ratio with parity, season and average temperature. There were also some significant results ($p<0.05$) for primi_multi, but only with two ratios: HD/ML and HD/FL (Table 5).

All ratios showed a tendency towards being significantly lower ($p<0.05$) with higher parities and, season 3 ratios were all significantly higher ($p<0.05$) when compared to the other two seasons.

Table 5. Summary of significant regression model results in combined data.

	Parity	Primi_multi	Season	Temp_avg
HC/ML	P<0.05	-	P<0.05	P<0.05
HC/FL	P<0.05	-	P<0.05	P<0.05
HD/ML	P<0.05	P<0.05	P<0.05	P<0.05
HD/FL	P<0.05	P<0.05	P<0.05	P<0.05
HG/ML	P<0.05	-	P<0.05	P<0.05
HG/FL	P<0.05	-	P<0.05	P<0.05

7. Discussion

The health, well-being and performance repercussions of metabolic stress, not only in the mother but in the calf as well, makes it a condition worth understanding in hopes of lessening its impact in dairy farming (Esposito et al 2014; Sheldon et al. 2018; Ling et al. 2018)

We saw in the studies performed on heat stress (Lamp et al. 2015; Koch et al. 2016) that at its core the physiological effects that carried over to the calves were all in an attempt to better protect them from a following exposure to the same environmental stimulus, thermal stress (the changes in metabolism and production occurred in order to support processes that produced less metabolic heat, favouring glucose oxidation over NEFA, for instance). It is not unreasonable to assume that carryover effects of metabolic stress are equally well founded. We hypothesized that calves born from metabolic stressed mothers, prioritize survival over production and, therefore, when faced with limited nutrient supplies, they will first employ them in the development of the organs that are vital for survival. In order to confirm said hypothesis, we expected that for a higher degree of metabolic stress, ratio values would increase due to a bigger relative size difference between the vital organs and the long bones (figure 20).

$$\frac{\uparrow measurement\ of\ vital\ organs\ (HC, HD\ or\ HG)}{\downarrow measurement\ of\ long\ bones\ (ML\ or\ FL)} = \uparrow ratio\ values$$

Figure 20. Relationship between the measurements and ratio values

As previously reviewed (Costa 2015; Sordillo 2016), certain groups are more vulnerable to the detrimental effects of metabolic stress (high yield dairy cattle, cattle with high and low BCS, gestating dairy heifers, cattle with twin pregnancies and heat stressed cattle). In this study, it was opted not to measure biomarkers such as NEFAs and BHB, opting instead to quantify the mothers metabolic stress based on how well they fit the criteria for these risk groups. This allowed us to reduce cow manipulation to the absolute minimum, preventing any additional stress to the late gestation cows, while simultaneously developing a simple method to replicate, that could easily be employed in several dairy farms by checking their logs.

The measurements chosen were based on pragmatism: how easy they would be to perform and replicate in standing living calves and how well they represented the prenatal development of the long bones, cranium and thorax. According to Lawrence and Fowler (2002)**,** bone measurements, are a good way of quantifying growth while reducing animal stress to a minimum and, in recent studies (Kamal et al. 2014; Beythien et al. 2017), similar measurements were used in newborn animals to determine fetal growth.

7.1. Portuguese data

As expected, significant differences in measurements were displayed based on gender. The size differences between males and females are common knowledge and, in this study, translated to significantly higher HD, WH and SW ($p<0.05$) in newborn male calves.

Parity presented few significant changes in the measurements. No studies were found on the effect of parity on neonatal measurements, however there are several that link a higher parity number with increasing birth weights (Legault and Touchberry 1962; Sieber et al. 1989; Kertz et al. 1997). In this study, the HD measurements were only significantly higher ($p<0.05$) in parity 6 calves, but trended higher for HD and HG for parity 4 calves and in the latter for parity 6 calves. Originally, parity 5 also showed significant differences in measurements, specifically to the HG and ML, however it was only one single calf and he skewed the results due to his abnormally small size, so he was removed as an outlier. We should consider that only a few calves had high parities (3, 1, 2 and 1 calves for parity 4, 5, 6 and 7, respectively) and therefore a bigger sample in these parities would probably evidence additional significant results.

There are discording opinions on whether milk production affects size at birth. Swali and Wathes (2006), for instance, observed significant correlations between milk output and HW, while other authors (Legault and Touchberry 1962; Zhang et al. 2002) found no evidence to support it. Likewise, the present study found no connection between the measurements and M305d, however we hoped that some changes to the ratios could be linked to this variable (more specifically, that increasing values of M305d could be connected to increasing values in ratios). Since no significant results were detected, we must consider the obvious possibility that our hypothesis is incorrect and milk output has no relation to allometric fetal growth but, we should also contemplate that these results came from a sample of only about 100 calves and that far larger samples could be necessary to evidence their relation. Another possibility is that the relation they have is not linear and that it is wrong to assume that an increase in one will lead to a proportional variation in the other. Instead, there might be a threshold after which the influence they have is always the same. In other words that after a certain level of milk output all calves could be similarly affected and therefore all exhibit similar changes in proportion.

In contrast to our own results, studies have shown that birth weight and size (Swali and Wathes 2007; Kamal et al. 2014) are lower in calves born from primiparous cows. For the ratios as well, no significant changes were found in them that could be linked to this variable. However, observational studies are prone to variation so it is likely that a larger sample size could evidence different results. Another possible explanation could be that the energy spent by the heifers for their own growth and development is equivalent to the energy spent in milk production by the

lactating cows and therefore the pressure they exert in the developing fetus ends up having a similar/equal effect.

Overall, these results suggest that neither parity nor lactation have any significant influence on calf size or proportion at birth, however the discrepancy with other studies means that further investigation should be done in order to determine if this occurs due to a wrong hypothesis or an insufficient sample size.

7.2. Belgian data

The Belgian data was analysed in much the same way as the portuguese one, yet it presented key differences in the results. Unlike the Portugal data and, despite testing a smaller number of calves, both parity and primi_multi displayed a significant influence ($p<0.05$) in ratio values. The fact that these two variables presented significant results to the same ratios (HD/ML, HD/FL, HG/ML and HG/FL) can be justified when we consider that the significantly higher ratio values were mostly from the first to the second parity and, therefore, simultaneously from a lower to a higher parity cow, and from a primiparous to multiparous cow. Even though this in accordance with the previously mentioned literature (Legault and Touchberry 1962; Sieber et al. 1989; Kertz et al. 1997; Swali and Wathes 2007; Kamal et al. 2014), it differs from the portuguese results, possibly leading to different conclusions, such as, that the energy necessary for lactation as a stronger influence on the fetus than the energy necessary for the growth and development of the gestating heifer. Additionally, the discrepancies in results between data sets (portuguese and belgian) also further emphasize the need for a larger sample size in order to draw any meaningful conclusions that might apply to the breed as a whole.

In the Belgian data two additional variables, season and average temperature, were also considered. Despite the small difference they show in temperature (4.5 ºC for season 1 and 5.3 ºC for season 2), significant changes were seen in the ratios based on season and, correspondingly, temp_avg. The calves born at temp_avg 5.3 ºC in season 2 had significantly higher ratios than those born at 4.5 ºC in season 1. This 0.8 ºC variation is not enough to explain the difference in ratios, nor is an average temperature of 5.3 ºC high enough to be considered heat stress, however without additional variables to justify them (for instance differences in nutrition or in managing practices), the reason for these results remains unclear.

7.3. Combined data

It is not uncommon for farm as a factor to influence the outcome a study. Bakir et al. (2004), for instance, connected significant changes in calf birth weight to different farms. Therefore, in

order to properly analyse the combined data of both countries, we first had to verify if we could process the complete data as a homogenous whole, or if there were any significant differences between the measurements taken in Portugal and in Belgium.

Variance analysis showed that there was a significant variation ($p<0.05$) in HC_ML values associated with farm. Further analysis to determine if this could be linked to any other specific variable showed that, the variation we saw for farm, was related to season. Taking that into account, we performed a second variance analysis of HC_ML, this time for season and searched for possible links to other variables. As expected, we also got a positive result ($p<0.05$), but no relationship could be established to any others. Seeing that, aside from season, no other variable justified the variation linked to farm, we performed a random effect analysis. However, even while using farm as a random effect, only season remained statistically significant.

Based on this information we can surmise that some of the variance between farms can be explained by the differences in season, but the variance associated with season cannot be justified by any other variable, raising the possibility that season is the only variable with any statistical weight. Not entirely unexpected, seeing as one of the major discrepancies between both data sets were the dates when they were performed (February to March in Belgium, and July to August in Portugal) and considering other studies (Sieber et al. 1989; Tao and Dahl 2013) that have shown that season and temperature have a major influence in calf size and weight at birth. Regardless, the reason why all the variation in the data can be explained by both farm and season is, most likely, because they influence each other and therefore it is very difficult to separate them and determine where the effects of one end and the other begin. That said, there are other possible explanations for these results: variables that were not quantified in this study, such as nutrition, maintenance conditions and farming practices, for instance, could also be responsible (Bakir et al. 2004).

Another explanation lies in the fact that body measurements, especially in animals, are typically prone to variation either due to the one who performs the measurements or the measuring technique itself. Despite the actions we took to circumvent this, standardizing the protocol as much as possible and having both performers practice the protocol together, the possibility of human error is always present.

Finally, there might also be a genetic component at work. Although all animals in the study were Holstein-Frisian cows, it is possible that, despite being the same breed, each country is selecting with different outcomes in mind, for different factors and aiming for a different type of animal.

The main reason we analysed the combined data was to determine if we could attribute any significant changes in ratio values to heat stress. Considering the differences in average temperature (4.5 ºC, 5.3 ºC and 25 ºC for season 1, 2 and 3, respectively) we hoped that ratios values in Portugal would be significantly higher than in Belgium. The results of the random effect analysis do in fact point towards that same conclusion. However, while it is possible that heat stress is responsible for the variation in ratios, we cannot say with definite certainty if those results were due to the differences in temperature or other unquantified variables.

Finally, we checked how each independent variable related to the ratios with regression models and evidenced that parity, primi_multi, season and temp_av all had a significant connection to them. Regretfully, since farm has such strong effect on the sample, we cannot give any significant weight to these results and only state that they allude to the fact that they may be related.

7.4. Considerations for further investigation and study limitations

The comparison between countries performed in the study was used to circumvent time limitations. Ideally, the effect of heat stress would have been quantified in one location by performing the same protocol over, at the very least, a whole year, giving us a better insight on how the changes in season influenced calf birth size, while eliminating any additional variables associated to the differences between farms. Furthermore, that timeline would have the added benefit of greatly increasing sample size, another of this study's main limitations. That said, there is still value in implementing the study and comparing results between different farms but, whenever possible, in should be performed by the same individual in order to completely remove any personal biases in the technique.

Aside from increasing sample size, there are other ways by which we might optimize this study. Such as the determination of BCS in relevant phases of the gestation (lactation peak, dry period, parturition) or perform a more precise determination of environmental and body temperature during gestation.

We should also consider that while avoiding additional stress to the mothers is important, no two animals are completely alike, and the same volume of milk production, or even NEB for that matter, might not influence metabolic stress to an equal degree in two different cows (Castillo et al. 2005; Kessel et al. 2008). The use of metabolic stress markers such as NEFAs and ketone bodies, could provide a lot of insight to the actual metabolic status of the cows, regardless of individual variation, and allows us to better correlate them to the risk factors in this study.

8. Conclusion

The metabolic status of dairy cattle is a complex and intricate topic. Many factors work together to keep metabolism running smoothly, but it is quite easy for it to go out of bounds.

Considering the growing awareness there is about fetal programming (Opsomer et al. 2017), looking past the implications these metabolic imbalances can have on the mother and focusing instead on asking what they can say about the future of their offspring's health, fertility and productivity, are becoming increasingly more meaningful questions. In this experimental study we strived to answer one such question: can metabolic stress "program" the calf to prioritize the development of specific vital organs in utero? Unfortunately, the conclusions we could draw were few but, still, not without merit. There were inconsistencies between the results we got from Portugal and the results we got from Belgium, while they did not contradict each other regarding the influence gender and parity have on calf birth size, the effects primiparity exerted showed different results. The fact that the exact same protocol gave us conflicting responses tells us that the results we got from these models may be premature and that larger samples may be necessary to showcase definitive answers.

On the other hand, when we looked at both datasets as a whole, the only thing we could say for certain was that season played a big role in how divergent the data from the Portuguese farm and the Belgian farms were, but the influence of other factors, such as nutrition, managing conditions, farming practices or person performing the study were not considered.

Although we could not determine what we set out to prove, the data collected was still useful and can serve as a baseline to develop similar protocols and as a starting point for future studies on the subject. It would be very interesting to see how a more rigorous protocol, performed on a larger sample over a longer period of time would change these results.

The mechanisms by which fetal development can influence an animal's adult life still have many unanswered questions, but we can and should keep trying to answer them, knowing that attaining a more complete understanding of their inner workings can be vital in allowing us to provide them a better quality of life, a healthier reproductive life and a greater capacity to sustain their productive life.

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Appendix I - Data collected in Portugal (July and August 2017)

Farm	Calf	Mother	Date of birth	Date of measurement	Sex	Height at withers (cm)	Diagonal Length (cm)	Chest circumference (cm)	Width of the back (cm)	Width of the shoulders (cm)	Head circumference (cm)	Head diameter (cm)	Length metatarsus (cm)	Length forearm (cm)	Milk production during 305d	Parity	Conception date
PT_farm	2164	8395	28-Jun	05-Jul	female	65	50.5	80	20	18	50	17	33	25	11581	6	21/09/2016
PT_farm	2165	912	29-Jun	05-Jul	female	68,5	60	79	17	15.5	54	16	34	24	11461	2	30/09/2016
PT_farm	2166	1369	02-Jul	05-Jul	female	75	69	81	17	16	52	14	28	22	0	1	08/10/2016
PT_farm	2167	1398	02-Jul	05-Jul	female	74	57.5	78	18	16	51	15	27	23	0	1	20/10/2016
PT_farm	2168	986	02-Jul	05-Jul	female	75	58	81	17.5	16	53	15	27.5	20	9091	2	28/09/2016
PT_farm	4558	904	02-Jul	05-Jul	male	74	60	78	18	16	51	17	27.5	20.5	10402	2	13/10/2016
PT_farm	4559	1034	03-Jul	05-Jul	male	76	60	76	16	14	47	14	27	21	9600	2	30/09/2016
PT_farm	4560	1361	03-Jul	05-Jul	male	74	68	78	19	18.5	53	17	26	21	0	1	06/10/2016
PT_farm	2169	934	04-Jul	05-Jul	female	73	58	75	16	14.5	51	14	32	25	10479	2	30/09/2016
PT_farm	2170	705	05-Jul	05-Jul	female	68,5	55	73	17	15.5	47	15	26	20	9900	2	03/10/2016
PT_farm	2171	705	05-Jul	05-Jul	female	77	61	68	16	15	45	15	26	19.5	9900	2	03/10/2016
PT_farm	4561	114	05-Jul	05-Jul	male	77	64	82	19	17	52	17	29	21.5	13172	4	17/10/2016
PT_farm	2172	542	06-Jul	13-Jul	female	76.5	66	78.5	20	18.5	49	16	28	21	11947	3	26/09/2016
PT_farm	2173	1429	06-Jul	13-Jul	female	79	67	82	22	18	49	16	31.5	23	0	1	03/10/2016
PT_farm	2174	8528	07-Jul	13-Jul	female	82	72	89	21.5	18	54	18	31	23	10773	6	16/09/2016
PT_farm	2175	1433	08-Jul	13-Jul	female	79.5	68	85	21	17	52	16	33	22.5	0	1	08/10/2016
PT_farm	2176	938	09-Jul	13-Jul	female	76	65	79	18	16	51	16	29	21	9918	2	07/10/2016
PT_farm	2177	1375	09-Jul	13-Jul	female	78	65	82	21	18	51	16	28	21.5	0	1	30/09/2016
PT_farm	2178	1367	11-Jul	13-Jul	female	78	63	78	19.5	18.5	51	16	30.5	22	0	1	13/10/2016

PT_farm	4568	776	12-Jul	13-Jul	male	75.5	61.5	79	18	16	52	16	29	21.5	8347	2	30/09/2016
PT_farm	4569	1383	12-Jul	13-Jul	male	79	61	83	20.5	18	55	17	30	23.5	0	1	07/10/2016
PT_farm	2179	975	13-Jul	13-Jul	female	82	66	85	21	19	53	17	32	24.5	11560	2	07/10/2016
PT_farm	2180	1045	13-Jul	13-Jul	female	76	66	78	19	16	49	15	27	21	11041	2	13/10/2016
PT_farm	4570	1416	13-Jul	13-Jul	male	75.5	63.5	80	20	17	50	16	30	23.5	0	1	09/10/2016
PT_farm	2181	1380	14-Jul	19-Jul	female	78	61	81	21.5	19	52	16	27.5	22	0	1	20/10/2016
PT_farm	4571	1422	14-Jul	19-Jul	male	78	66	84	22	19	50	17	29.5	22	0	1	08/10/2016
PT_farm	4572	1347	15-Jul	19-Jul	male	81	64	88	22	19.5	52	18	33.5	25.5	0	1	06/10/2016
PT_farm	4573	1420	15-Jul	19-Jul	male	78	64	86	19	17	53	17	32	24	0	1	06/10/2016
PT_farm	2182	1428	16-Jul	19-Jul	female	73.5	63	78	19	17	48	16	32	25	0	1	25/10/2016
PT_farm	4574	1125	16-Jul	19-Jul	male	77	66	83	20	16	52	17	31	23.5	7711	2	13/10/2016
PT_farm	2183	596	17-Jul	19-Jul	female	76.5	70.5	84	20	16	54	17	27.5	22	12699	3	09/10/2016
PT_farm	2184	1357	18-Jul	19-Jul	female	77	60.5	79	21	17	53	16	30	23	0	1	18/10/2016
PT_farm	2185	1116	19-Jul	19-Jul	female	80	66	84	19	17	53	17	34	25.5	8849	2	10/10/2016
PT_farm	2186	1000	19-Jul	19-Jul	female	79	63	84	18.5	17	55	16	31	25	10104	2	07/10/2016
PT_farm	2187	1033	19-Jul	19-Jul	female	78	67	82	20.5	17	51	16	30	23	9963	2	14/10/2016
PT_farm	2188	1450	19-Jul	19-Jul	female	77	68	80	19	17	53	17	30	24	0	1	03/11/2016
PT_farm	2189	1447	19-Jul	19-Jul	female	74	61.5	76	18.5	17.5	49	16	27	22	0	1	13/10/2016
PT_farm	2190	570	19-Jul	19-Jul	female	78	64	83	21.5	19	55	16	32.5	24	12146	3	16/10/2016
PT_farm	4575	8313	19-Jul	19-Jul	male	77	66	82	21	20	53	16	32.5	25	11470	7	09/10/2016
PT_farm	4576	995	19-Jul	19-Jul	male	80	72	81.5	20	19	53	17	32.5	24.5	11698	3	08/10/2016
PT_farm	4577	981	19-Jul	19-Jul	male	79.5	68	81	20	19	52	17	30.5	24	10886	2	12/10/2016

PT_farm	2191	1368	20-Jul	25-Jul	female	78	64	85	21.5	19	51	17	34.5	26	0	1	22/10/2016
PT_farm	4578	201	20-Jul	25-Jul	male	79.5	74	86	20	19	51	17	31.5	25	10591	4	14/10/2016
PT_farm	4579	843	20-Jul	25-Jul	male	79	69.5	84	21	19.5	50	17.5	29.5	25.5	10935	2	09/10/2016
PT_farm	4580	1013	20-Jul	25-Jul	male	79	65	82	20	18.5	51	16	33	25.5	11275	2	13/10/2016
PT_farm	2192	924	21-Jul	25-Jul	female	79.5	64.5	83.5	21	20	54	17	30	23.5	8275	2	14/10/2016
PT_farm	2193	9030	22-Jul	25-Jul	female	77.5	67.5	80	20	18.5	54.5	16	21	25	9965	5	16/10/2016
PT_farm	2194	1381	22-Jul	25-Jul	female	76	62.5	79	19	17.5	49	15.5	29.5	22	0	1	18/10/2016
PT_farm	4581	548	22-Jul	25-Jul	male	78.5	63	81	21	19.5	48	17	29.5	23	10145	3	23/10/2016
PT_farm	4582	1391	22-Jul	25-Jul	male	62.5	53.5	64.5	16	15	47	14.5	24.5	19.5	0	1	16/11/2016
PT_farm	4583	985	23-Jul	25-Jul	male	78	67	81	20.5	19	55	17	30.5	24.5	9659	2	17/10/2016
PT_farm	4584	921	24-Jul	25-Jul	male	79	68	80	20	19	51	16	32	25.5	10086	2	22/10/2016
PT_farm	4585	1418	24-Jul	25-Jul	male	78	65	79.5	20	19	51.5	16	30	24.5	0	1	20/10/2016
PT_farm	4586	1468	24-Jul	25-Jul	male	75	61	79	18.5	18	49	15	27.5	20.5	0	1	05/11/2016
PT_farm	2195	1417	26-Jul	01-Aug	female	79	68	78	19.5	17.5	51	16	30.5	24	0	1	20/10/2016
PT_farm	4587	1005	27-Jul	01-Aug	male	79	67	83	20.5	17.5	52	16.5	30.5	23	10570	2	28/10/2016
PT_farm	2196	1334	28-Jul	01-Aug	female	77	68	80	19.5	17.5	49	16.5	30	22.5	0	1	24/10/2016
PT_farm	4589	1412	28-Jul	01-Aug	male	79.5	67.5	81	22.5	19.5	51	16.5	29	22	0	1	01/11/2016
PT_farm	2197	1442	29-Jul	01-Aug	female	75.5	65	79	20.5	18.5	52.5	16.5	30	25.5	0	1	22/10/2016
PT_farm	2198	1086	29-Jul	01-Aug	female	77	69	81	20	17.5	52	16	31	25.5	10682	2	22/10/2016
PT_farm	4590	1410	29-Jul	01-Aug	male	75	63	76	20.5	19	52	15.5	29.5	23.5	0	1	01/11/2016
PT_farm	4591	1452	29-Jul	01-Aug	male	75	67	80	20	18	47	15.5	28.5	23	0	1	25/10/2016
PT_farm	4592	1452	29-Jul	01-Aug	male	76	63	82	21	19.5	52	15.5	29	25	0	1	25/10/2016

PT_farm	2199	1011	30-Jul	01-Aug	female	74.5	65	79	20.5	17.5	52	16	29	25.5	11400	2	24/10/2016
PT_farm	4593	480	30-Jul	01-Aug	male	81	67.5	86	21.5	20	54	17	30.5	24	12402	3	23/10/2016
PT_farm	2200	968	31-Jul	01-Aug	female	76	69	80	20	17	53	16	31.5	24	11002	2	21/10/2016
PT_farm	4594	1472	31-Jul	01-Aug	male	81.5	67.5	82	21.5	20	54	17	30	25.5	0	1	27/10/2016
PT_farm	4595	989	31-Jul	01-Aug	male	78	65	79	19	17	51	16	27.5	23	10478	2	29/10/2016
PT_farm	4596	942	31-Jul	01-Aug	male	78	69	80	20	18.5	53.5	17	28.5	22	10939	2	28/10/2016
PT_farm	4597	1105	31-Jul	01-Aug	male	80	64	84	21	18	53	16.5	30	25	10042	2	24/10/2016
PT_farm	2201	1049	02-Aug	09-Aug	female	72	62.5	73	18	16.5	47	16	27.5	22	10414	2	08/11/2016
PT_farm	4599	1360	02-Aug	09-Aug	male	77.5	64	83	20	18.5	52	16	31	25	0	1	01/11/2016
PT_farm	4600	624	03-Aug	09-Aug	male	78	71	85	22	22	52	16	30.5	24.5	10891	3	27/10/2016
PT_farm	4601	580	04-Aug	09-Aug	male	77.5	67.5	80	21	19.5	50	16	31	24.5	11450	3	02/11/2016
PT_farm	4602	1446	04-Aug	09-Aug	male	80	70	81.5	21.5	20.5	53	16	30.5	23	0	1	01/11/2016
PT_farm	4603	1440	05-Aug	09-Aug	male	75	64	80	19	18	49	15.5	30.5	24.5	0	1	10/11/2016
PT_farm	2202	662	05-Aug	09-Aug	female	74	68.5	80	20	18	53	16	30	24	10826	3	06/11/2016
PT_farm	4604	1107	05-Aug	09-Aug	male	82.5	73	82	20	17.5	52.5	15.5	32.5	26	9765	2	27/10/2016
PT_farm	2203	491	05-Aug	09-Aug	female	76	66.5	80	20	18.5	49	16	30	24	12165	3	10/11/2016
PT_farm	2204	1478	06-Aug	09-Aug	female	76	66	80	20	19	49.5	16	30.5	24.5	0	1	01/11/2016
PT_farm	4605	1436	06-Aug	09-Aug	male	78	68	81.5	21	20	53	16	30	26	0	1	03/11/2016
PT_farm	2205	1004	06-Aug	09-Aug	female	81	70	84	20.5	19.5	51.5	16	31.5	26.5	10629	2	05/11/2016
PT_farm	4606	643	07-Aug	09-Aug	male	74	64.5	76	19.5	18	51	15.5	30	24	12799	3	08/11/2016
PT_farm	4607	643	07-Aug	09-Aug	male	73.5	63	77	20	18	51	15	29.5	24.5	12799	3	08/11/2016
PT_farm	4608	1480	07-Aug	09-Aug	male	75.5	65	79	20	18.5	51	15	32	25.5	0	1	09/11/2016

PT_farm	4609	9180	08-Aug	09-Aug	male	78	67	84	21	20	53	16.5	31.5	25.5	10756	4	03/11/2016
PT_farm	2206	961	09-Aug	09-Aug	female	72.5	65	75	19	17	48	15	29	23	10042	2	12/11/2016
PT_farm	2207	1124	10-Aug	16-Aug	female	73	62	76	19	17	47	15	29	22.5	8650	2	12/11/2016
PT_farm	4610	594	10-Aug	16-Aug	male	78.5	65	81	21	19	49	16.5	30	24.5	10428	3	09/11/2016
PT_farm	4611	9068	11-Aug	16-Aug	male	81	73.5	86.5	22	20.5	54	16	32	27.5	10111	5	03/11/2016
PT_farm	4612	1439	11-Aug	16-Aug	male	75	63.5	84	21.5	19	52	16	29.5	24.5	0	1	05/11/2016
PT_farm	2208	1477	12-Aug	16-Aug	female	75	66	79	19.5	18.5	51	15.5	30	26	0	1	09/11/2016
PT_farm	2209	505	13-Aug	16-Aug	female	78	70	80.5	21	18.5	48.5	16	31.5	26	10987	3	11/11/2016
PT_farm	2210	479	14-Aug	16-Aug	female	77	64	82	21	18	51.5	15.5	31	27	11650	3	07/11/2016
PT_farm	4614	1378	14-Aug	16-Aug	male	78	63.5	79	20	19	51	16	31	25	0	1	16/11/2016
PT_farm	4615	817	15-Aug	16-Aug	male	77	60	77	19	12	51	15.5	28.5	23.5	10423	2	03/11/2016
PT_farm	4616	1134	15-Aug	16-Aug	male	79	68.5	82.5	21	20	55	17	32	26	9529	2	14/11/2016
PT_farm	4617	641	15-Aug	16-Aug	male	79	72	82	20	19	53	16	31	27	11224	3	17/11/2016
PT_farm	4618	641	15-Aug	16-Aug	female	74	61	72	18	16	46	15	28	22	11224	3	17/11/2016
PT_farm	2212	1040	16-Aug	16-Aug	female	80	69	82	20	17.5	52.5	16	30	24	11089	2	10/11/2016